INTER-PHYLOGENETIC SPECIFICITY IN THE BONDING OF AMINO ACIDS TO tRNA[1]

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The first step in protein synthesis is generally considered to be the reaction of a free amino acid (AA) with adenosine triphosphate (ATP) and an appropriate "activating" enzyme to form an "activated" amino acid (probably 5′-aminoacyl adenylate; AA ~ AMP) and pyrophosphate (Hoagland, Zamecnik and Stephenson, 1957).

This activated amino acid reacts with the 2′ or 3′ OH of a low molecular weight ribonucleic acid (tRNA) to release AMP and produce an aminoacyl tRNA which subsequently transports the amino acid into the appropriate part of the protein being synthesized. Evidence for this unique RNA was adduced more or less simultaneously and independently by Holley (1957) and by Hoagland *et al.* (1957), as well as indirectly by Hultin (1956) and Hultin and Beskow (1956). The structures of several tRNA's have been determined and it seems quite likely that more than one tRNA accepts a particular amino acid, although there is only limited evidence that more than one aminoacyl tRNA ligase exists for each amino acid (Yu, 1966; Barnett, Epler and Brown, 1967).

It is presumed that some sort of base pairing of the aminoacylated tRNA with genetic nucleic acid (mRNA, virus RNA or even DNA) must account for the extraordinary precision with which amino acids are assembled into proteins (Loftfield, 1963) or with which phenotypes reflect different genotypes. There is some evidence that the genetic code is universal and corresponding evidence that the insertion of an amino acid into a particular locus depends only on the tRNA to which it is attached. Berg and Ofengand (1958) showed that the activating enzymes also catalyzed the transfer of amino acid to tRNA. Hecht, Stephenson and Zamecnik (1959) showed that most, if not all, tRNA's terminated in an adenylic-cytidylic-cytidylic trinucleotide and several recent studies on structure show that the secondary structures may be similar for all tRNA's. It was the purpose of this work to establish whether activating enzymes from widely different sources could aminoacylate tRNA's from other phyla. Although the results are not gratifyingly unequivocal, we find that interaction between heterologous pairs exists where others have found it absent. In some cases, the interaction of heterologous pairs of tRNA and enzyme, as measured by our methods, is better than between homologous pairs. Our techniques show that the aminoacyl tRNA is chemically active however obtained, and that there is no chemical evidence of a multiplicity of aminoacyl tRNA's.

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Materials and Methods

(A) L-1-[^{14}C]-valine and L-1-[^{14}C]-leucine (20 c.p.m. per $\mu\mu$mole) were prepared by the Bucherer hydantoin synthesis (Loftfield and Eigner, 1966). TRIS (tris (hydroxymethyl)-aminomethane), ATP (adenosine triphosphate), and other reagents were purchased from commercial sources.

(B) Preparation of enzymes—*E. coli.* Ten g. of *E. coli* strain B cells were suspended in 50 ml. of .02 *M* TRIS buffer pH 7.5 containing 600 mg. of reduced glutathione per liter. The cells were ruptured by high pressure passage through the orifice of a Ribi cell fractionator. The suspension was then centrifuged two hours at 27,000 *g*. The supernatant fluid possessed a high concentration of both valine and leucine tRNA ligases and was generally used as such. Occasionally, the enzymes were further purified by column chromatography on DEAE cellulose (Berg, Bergmann, Ofengand and Dieckmann, 1961).

Toadfish. One gram of toadfish liver was homogenized in a Potter-Elvehjem homogenizer with 1 ml. of Medium A (0.35 *M* sucrose, 35 m*M* $KHCO_3$, 4 m*M* $MgCl_2$ and 25 m*M* KCl). The suspension was centrifuged at 24,000 *g* for 30 minutes. The supernatant phase was removed and the sediment extracted with a second 1-ml. portion of Medium A. All of the valine and leucine activating enzymes appeared in the supernatant phases which were used as such.

Starfish. The gonads, male or female, appeared to be the best source of amino acid activating enzymes. Ripe gonads were minced and stirred gently one-half hour in fresh sea water to release eggs or sperm. The water with eggs or sperm was decanted and the suspension of gonad tissue was homogenized in a Potter-Elvehjem apparatus with an equal volume of Medium A containing 600 mg./l. of reduced glutathione. The suspension was centrifuged 10 minutes at 20,000 *g* and the supernatant phase was used as the enzyme source.

Yeast. Fresh bakers yeast was cultured and harvested in log phase by Dr. J. Scaletti of The University of New Mexico. Fifty ml. of packed cells were suspended in 100 ml. of 0.02 *M* pH 7 phosphate buffer containing 600 mg./l. of reduced glutathione and forced through a Ribi press at 35,000 lbs./in.^{2} The suspension was centrifuged 20 minutes at 20,000 *g* and $(NH_4)_2SO_4$ was added to 80% saturation. After 20 minutes at 4° C., the precipitated protein was collected by centrifugation at 20,000 *g* for 20 minutes. The precipitate was dissolved in 5 ml. of the above buffer, 1 g. of Bentonite was added and the slurry was allowed to stand at room temperature 30 minutes. The Bentonite was removed by centrifugation at 20,000 *g* and the enzyme solution was dialyzed against the phosphate glutathione buffer for six hours at 4°. The RNA contamination could be reduced by treating each ml. of the enzyme solution with 10 μmoles of $MgCl_2$, 600 μg. of glutathione and 10 mg. of streptomycin for 30 minutes at 0°. The precipitate was removed by centrifugation and the solution again dialyzed against the phosphate glutathione buffer.

Each enzyme preparation was routinely examined for activity using the [^{14}C]-amino acid hydroxamate technique (Loftfield and Eigner, 1963).

(C) tRNA's.—*E. coli* tRNA and yeast tRNA were gifts from Schwartz Bioresearch.

Starfish.—Eighty ml. of ripe starfish eggs were suspended in 80 ml. of 0.001 *M* pH 7.5 TRIS buffer together with 16 ml. of 0.1 *M* $MgCl_2$. The suspension was

homogenized at 0° for 5 minutes in a Waring Blendor. Undissolved material was removed by centrifugation for 10 minutes at 16,000 *g*. The supernatant phase was gently mixed for one hour at room temperature with an equal volume of 90% phenol. Centrifugation separated the mixture into two phases. To the upper phase were added 0.1 volume of 20% potassium acetate and 2.5 volumes of ethanol. After 16 hours at −10°, the precipitate was collected by centrifugation, washed once with 67% ethanol and drained. The precipitate was then suspended in 1 *M* NaCl and centrifuged at 15,000 *g* for 30 minutes to sediment most of the ribosomal RNA. The supernatant was dialyzed against water, treated with 0.5 *M* TRIS at pH 8.8 for 45 minutes to hydrolyze aminoacyl tRNA, neutralized with acetic acid and dialyzed again. Although the solution showed a spectrum typical of RNA, it was very viscous, suggesting the presence of a highly polymerized contaminant. Therefore the solution was stirred two minutes at 20° with one volume of 2.5 *M*, pH 7.5 potassium phosphate buffer and one volume of methyl cellosolve. The mixture was separated into two phases by centrifugation at 15,000 *g* for 10 minutes at 4°. tRNA was precipitated from upper phase by adding potassium acetate and alcohol as above.

Toadfish.—Forty grams of toadfish liver were homogenized and worked up in much the same way as the starfish eggs. Although the product had a slight gray-brown color, it had an ultraviolet spectrum that was typical of tRNA. Like each of the other tRNA's prepared, the ratio of $O.D._{260}/O.D._{280}$ was approximately 2.0.

Using homologous enzymes, the maximum aminoacylation of each tRNA was: in mμ moles per mg.; *E. coli*, leucine 1.0, valine 1.2; yeast, leucine 0.37, valine 0.35; starfish, leucine 0.45, valine 0.45; toadfish, leucine 0.59, valine 0.36.

(D) The aminoacylation of tRNA was carried out as previously described (Loftfield and Eigner, 1967a) and as summarized in the legend of Figure 1.

Results

There has been an abundance of work on the cross-reaction of activating enzymes and tRNA's of different species. For the most part only bacterial, yeast and vertebrate sources have been used and examination has been confined to a few amino acids. In general, there seems to have been some cross-reaction when yeast or vertebrate sources of enzyme and RNA were used but none or little when bacteria provided one of the ingredients. Benzer and Weisblum (1961) showed that the extent of cross-reaction varied with the amino acid used. We (Loftfield and Eigner, 1963) demonstrated that if sufficient care is taken, pairs reported to have no cross-reaction can be shown to react, though the rates may be less than 1% of the homologous pairs.

Stulberg and Novelli (1962; p. 422) correctly point out some of the errors in making hasty statements that interspecies interaction does not obtain. Frequently so-called "tRNA" has not been shown to accept amino acids from the homologous enzyme. Thus many of our preparations of starfish "tRNA" had the physical and chemical properties of tRNA without being able to bind covalently significant amounts of several amino acids even using starfish enzyme. Other workers have occasionally reported no effort to determine whether the aminoacylated tRNA was biochemically or chemically active.

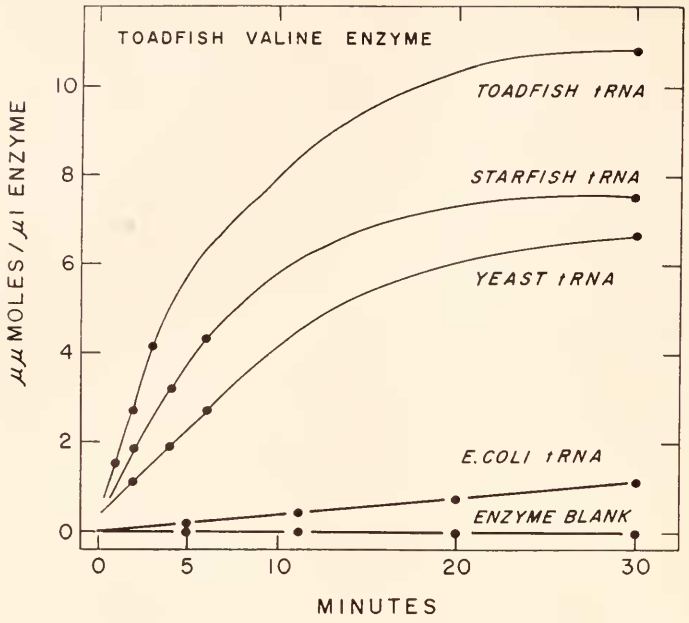

FIGURE 1. A typical experiment that shows the rate of valyl tRNA formation by the toadfish valine activating enzyme with four different species of tRNA. The reactions were carried out at 25° with 10 m*M* ATP, 0.1 *M* TRIS (pH 7.5), 30 μ*M* [^{14}C]-valine, 0.5 mg. tRNA per cc., and 6 μl. enzyme solution per tube (final vol. per tube 225 μl.; four aliquots of 50 μl. each were taken at times indicated). The enzyme blank contains everything except added tRNA. The aliquots were worked up as previously described (Loftfield and Eigner, 1967a). The 30-minute observations correspond to the incorporation of 0.35 mμ mole of valine to 1 mg. of yeast tRNA and to 0.45 mμ mole of valine into 1 mg. of starfish tRNA, exactly the same saturating values as found with homologous enzyme. On the other hand, the *E. coli* tRNA is only about one-tenth saturated.

Hence the current work has been undertaken with considerable care to establish that aminoacylation of tRNA was actually taking place. Every figure noted in Table I represents a rate of reaction drawn from at least two different experiments in which the extent of reaction had been determined at no fewer than three time intervals. Figure 1 illustrates the kind of data used and the difficulties involved in comparing rates. For instance, many of our enzyme preparations contained an RNase which slowly destroyed the substrate RNA; this was clearly a greater problem if the reaction was slow or if larger amounts of enzyme solution were being used. Thus in some heterologous reactions similar to Figure 1, the tRNA was never saturated with amino acid. However, the addition of ATP and enzyme homologous with the tRNA at 30 minutes was ineffective. The receptor competence of the tRNA had been destroyed during the incubation. This was especially evident when enzyme and tRNA were pre-incubated without the amino acid. Depending on the species and preparation, the competence of the tRNA to accept amino acid steadily deteriorated. No two preparations of tRNA accepted, even from homologous sources, the same amount of leucine or valine and we (Loftfield

and Eigner, 1963, 1965) have previously shown that either aminoacylated tRNA or damaged (oxidized) tRNA inhibits the reaction. Even products of tRNA digestion have been shown to be inhibitory (Hayashi and Miura, 1966; Letendre, Michelson and Grunberg-Manago, 1966). We have presented in Table I relative initial rates of reaction where destruction of substrate, product inhibition and enzyme inactivation are minimized.

With due regard for all these difficulties, Table I contains material of interest. In keeping with earlier observations, most *E. coli* enzymes of established activity interact poorly if at all with non-bacterial tRNA's of established competence while there is a fair to better than natural interspecies reaction when the enzymes and tRNA's are derived from yeast or the higher organisms. (At best, this is only a weak generalization; starfish valine enzyme reacts poorly if at all with *E. coli* tRNA while the leucine enzyme of starfish is as good as the *E. coli* enzyme.)

TABLE I

Relative rates of homologous and heterologous enzymic formation of aminoacyl tRNA's. The rates are expressed as per cent of the rate obtained for homologous tRNA and enzyme under standard conditions

Enzyme		tRNA			
		E. coli	Toadfish	Starfish	Yeast
E. coli	val	100	2	1.5	0.5
	leu	100	<0.1	<0.5	2
Toadfish	val	3	100	50	27
	leu	<0.1	100	47	3
Starfish	val	<1.	100	100	83
	leu	100	40	100	40
Yeast	val	40	200	140	100
	leu	45	300	65	100

Most striking is the observation that yeast enzymes specific for both valine and leucine are more active towards starfish and toadfish tRNA than they are towards yeast tRNA. Equally notably, yeast enzymes for both amino acids are quite active in transferring leucine or valine to *E. coli* tRNA while *E. coli* enzymes are almost inert towards yeast tRNA. Partly to the contrary, starfish enzyme is very active towards *E. coli* tRNA only with leucine while *E. coli* enzyme is poor in transferring either leucine or valine to starfish tRNA.

There is a concern whether some tRNA normally specific for one amino acid is being aminoacylated by another. Although there are several reports of multiple tRNA's specific for a single amino acid there are to date, only a few suggestions of a multiplicity of the aminoacyl tRNA ligases (Yu, 1966; Barnett *et al.*, 1967). Nonetheless we deliberately used crude enzyme preparations rather than purifying the enzyme so as to lose no active component. If our enzymes were transferring amino acids indiscriminately to a variety of the heterologous tRNA's, we might

expect that some tRNA's would accept more leucine or valine than they did from the homologous enzyme. In every case, we found that the heterologous aminoacylation yielded only the same extent of reaction as the homologous. (Note the legend in Figure 1.) No evidence was found to indicate that the heterologous reaction was less specific than the homologous reaction.

The most valid criterion of whether a heterologously aminoacylated tRNA was identical with the same tRNA aminoacylated with a homologous enzyme would be to compare the behavior of the two tRNA's in the synthesis of a specific protein. Unfortunately all cell-free syntheses of protein are suspect. The use of either natural messenger RNA (*i.e.*, to synthesize hemoglobin or β-galactosidase) or so-called synthetic mRNA's like poly-uridylic acid (to synthesize polyphenylalanine) leads to data in which high backgrounds, autolysis, RNase activity, salt

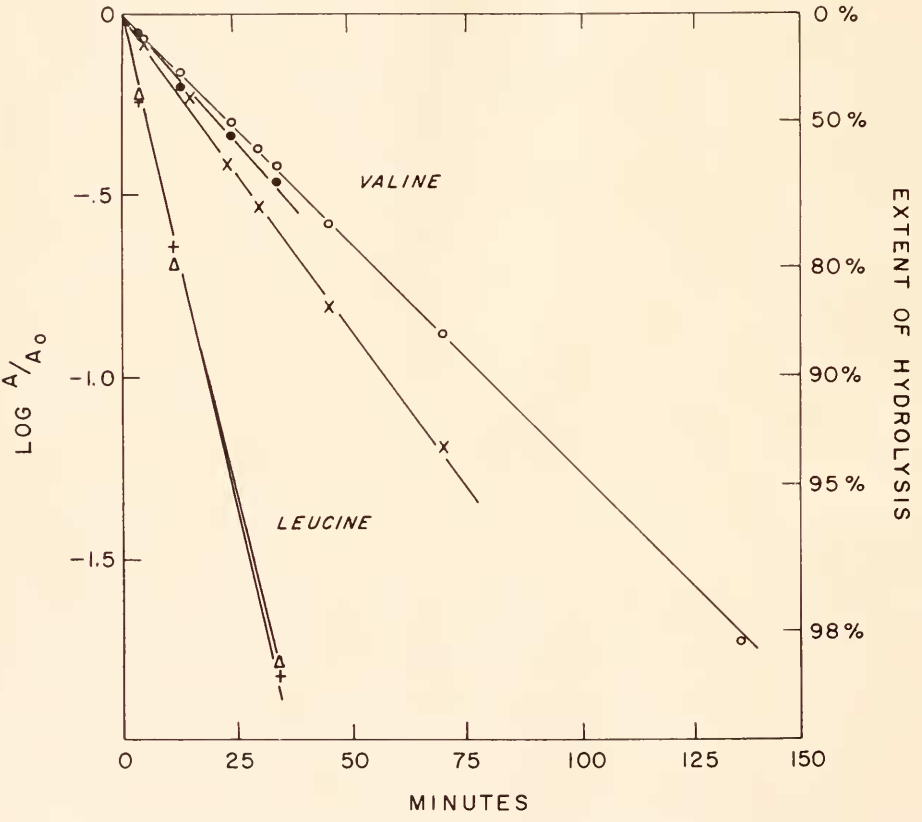

FIGURE 2. Rate of stripping of [^{14}C]-valyl and [^{14}C]-leucyl tRNA. The tRNA was labeled completely in each case at 25°, then made 0.16 *M* in Na_2CO_3 (pH 10.3) and brought to 35° to initiate the stripping reaction. Other conditions same as Figure 1, but larger amounts of enzyme were used. [^{14}C]-leucine was used with *E. coli* enzyme and *E. coli* tRNA (△), and toadfish enzyme and toadfish tRNA (+). [^{14}C]-Valine was reacted with *E. coli* enzyme and *E. coli* tRNA (○), toadfish enzyme and toadfish tRNA (×), and toadfish enzyme and starfish tRNA (●).

concentrations, etc., are prominent contributors to an ambiguous interpretation. Accordingly, we have used a much simpler criterion of the nature of the aminoacyl tRNA bond. Raacke (1958) pointed out that, although aminoacyl tRNA compounds are essentially simple esters, they are far more reactive than the aliphatic esters of amino acids. We chose to measure the rate of deaminoacylation of the aminoacyl tRNA's formed by either homologous or heterologous enzymic action. Figure 2 is illustrative of the kind of data obtained at pH 10.3 for a variety of these hydrolyses.

The first thing to be noted in Figure 2 is that the first order rate of hydrolysis is absolutely constant, *i.e.*, the semi-logarithmic plot shows no deviation from linearity. In every case, the hydrolysis was followed as far as the available [^{14}C]-aminoacyl tRNA permitted. Thus in the case of *E. coli* valyl tRNA the rate of hydrolysis remains constant until 97% complete hydrolysis. In no case is there evidence of a change in rate which certainly would have resulted from differences in chemical activities of the several leucyl tRNA's or valyl tRNA's. The graph shows clearly that the technique is sensitive to differences in rate of as little as 10%. Raacke showed that amino acids esterified to positions other than the unique 2′ or 3′ adenosyl terminal positions of tRNA would be a hundred times less reactive. Correspondingly, our data show that no more than 3% of the valine or leucine is bound to a relatively unreactive position such as a 5′ hydroxyl or one of the amino or enol residues. Whether the tRNA has been aminoacylated by homologous or heterologous enzyme, the rates of hydrolysis are well within the range established for a particular amino acid. It is noteworthy that the leucyl derivatives are some four times more labile than the valyl derivatives. This agrees with the well recognized differences in rates of hydrolysis of leucyl and valyl peptides and is consistent with the steric inhibition generalizations of Newman (1950). In fact, the relative rates of hydrolysis of the leucyl esters are somewhat less than would have been anticipated from the application of Newman's "Rule of Six" to the straightforward attack of a hdyroxyl ion or other nucleophile on the carbonyl bond.

Particularly to be noted are the clearly discernible differences in rates of hydrolysis of the several valyl tRNA's. Although these are much more stable than the leucyl tRNA's, the analysis is sensitive enough to detect differences which presumably result from differences in the structure of the tRNA.

The extraordinary activity of the yeast enzyme towards toadfish tRNA warranted a closer examination. It is quite conceivable that the high relative rates of aminoacylation are a consequence of our failure to saturate the homologous enzyme tRNA pairs. For instance if the K_m for binding toadfish tRNA to yeast enzyme is very low while the K_m for binding the yeast tRNA to yeast enzyme is high, we might expect that we should observe nearly maximal aminoacylation rates with toadfish tRNA while we were still observing only a fraction of the V_{max} with yeast tRNA. Figure 3 shows that this interpretation is quite incorrect. An Eadie plot of rate data collected at various concentrations of tRNA shows that the K_m for yeast valine tRNA on yeast enzyme is 2.2×10^{-7} *M* while the K_m for toadfish tRNA is 1.9×10^{-6} *M*. There is probably a ten-fold poorer binding of the toadfish tRNA which is more than compensated for by a six-fold increase in the reactivity of the toadfish tRNA as measured by V_{max}. If the observations of

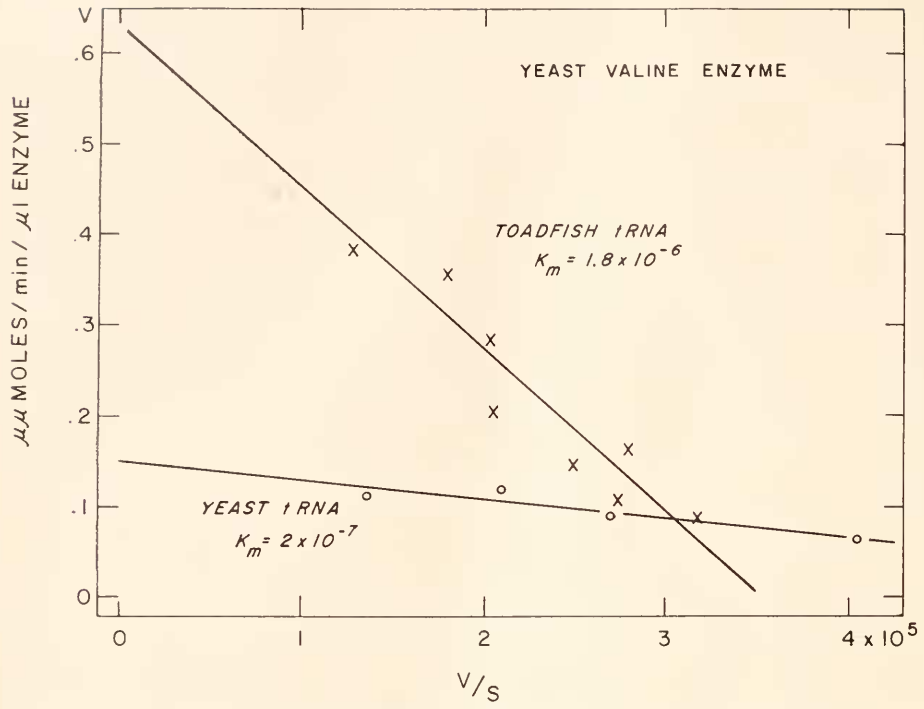

FIGURE 3. Eadie plot of formation of [^{14}C]-valyl tRNA by the yeast valine enzyme. The graph shows that yeast tRNA (○) has a K_m of 2×10^{-7} *M* and a V_{max} of 0.16 μμmoles/min./μl. enzyme. Toadfish tRNA (×) has a K_m of 1.8×10^{-6} but a V_{max} of 0.63 μμmoles/min./μl., four times greater than the V_{max} of yeast tRNA with yeast enzyme. Conditions as in Figure 1, except yeast tRNA varied from 0.4 to 2.0 mg./cc. and toadfish tRNA varied from 0.46 to 4.9 mg./cc.

Table I had been made with tRNA concentrations of 5 mg. per ml., the toadfish tRNA would have appeared to be three or four times more active than yeast tRNA.

DISCUSSION

It has been noted by ourselves and others that there is inter-species interaction of tRNA and the corresponding ligase. It has previously been thought that most non-bacterial tRNA's would interact with other non-bacterial enzymes while bacterial tRNA would not react with non-bacterial enzymes. We (Loftfield and Eigner, 1963) have previously shown some cross reaction between *E. coli* tRNA or activating enzymes and the tRNA or enzymes derived from yeast and ascites tumors even when the rates of aminoacylation were extremely low. The present observations extend the generalization to enzymes and tRNA's from four widely differing sources. As noted previously, the failure to observe interaction as measured by an immeasurably low reaction rate does not mean that there is no interaction. Binding of the heterologous tRNA to enzyme may occur, but may be in

an unproductive mode or the rate of reaction may be so low as to escape observation in the presence of adventitious ribonucleases.

Table I shows that either valine or leucine reacts measurably for every pair of enzymes and tRNA's examined. There is no obvious pattern as to which amino acid is more likely to cross-react. For instance, *E. coli* enzyme puts more valine than leucine onto toadfish and starfish tRNA but more leucine onto yeast tRNA.

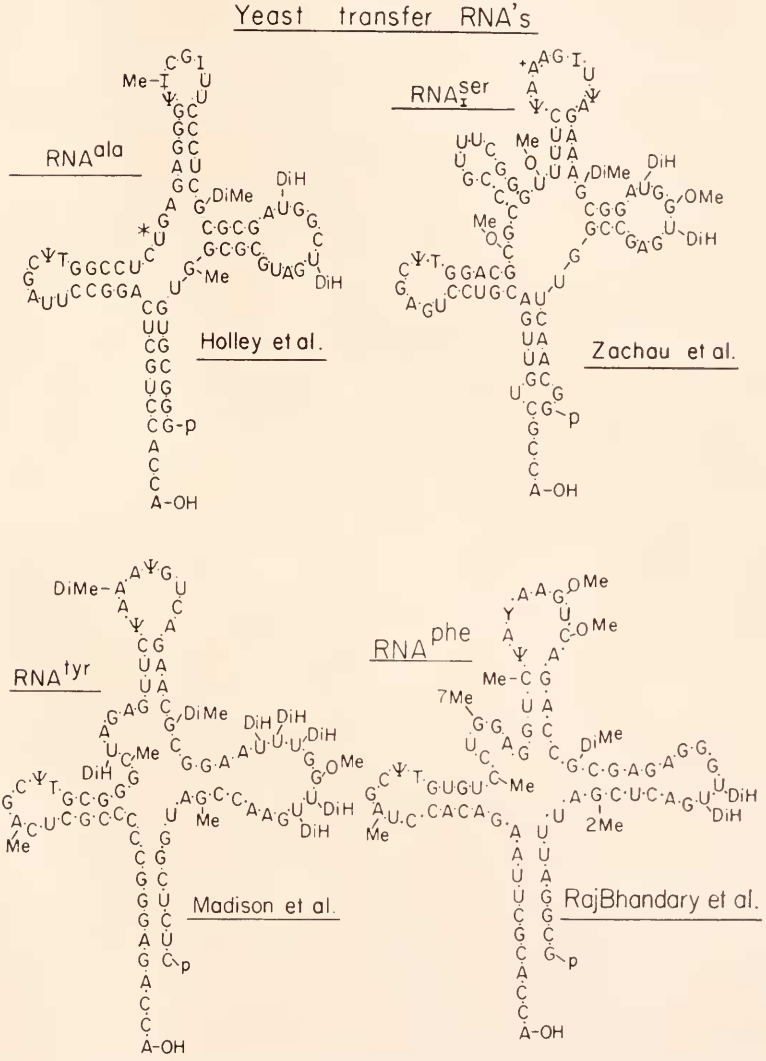

FIGURE 4. Possible conformations of four of the five tRNA's of known sequence. The sequences at the bottom (—OH end) and on the left loop are identical or very similar. The other portions, especially the right loop and the upper loop (which contains the presumptive anti-codon), are very unlike and may be responsible for the interphylogenetic amino acid specificity of the tRNA-ligase interaction.

Previously we have shown a similar irregularity in the actual K_m's for the binding of yeast or *E. coli* tRNA's to valine activating enzymes. In the present study this observation too is repeated and extended. Toadfish valine tRNA is bound to yeast enzyme ten times more weakly than yeast tRNA but is apparently many times more reactive once on the enzyme.

Beyond this, we have made no observations that would suggest any heterogeneity in the formed aminoacyl tRNA. In no case did the heterologous reaction proceed further than the homologous and in every case the rate of hydrolysis of the product followed first order kinetics for a homogeneous material as far as the reaction could be followed.

All these observations are consistent with the view that all valine specific tRNA's share some features in common which are not present in tRNA's specific for other amino acids. It would be easy to imagine that this common feature is the anti-codon specific for valine. However, there is no evidence that this is the case. Each tRNA molecule possesses about 80 nucleotides so there is abundant possibility for the existence of many other highly specific combinations of nucleotides. It should be noted that five yeast tRNA's have had their structures completely elucidated (alanine, Holley *et al.*, 1965; serine I and II, Zachau *et al.*, 1966; tyrosine, Madison *et al.*, 1966; phenylalanine, RajBhandary *et al.*, 1966). In every case, the suggested anti-codon contains one of the so-called minor bases and is very remote from the adenosine residue which is to be aminoacylated. Moreover, among the four sequences shown in Figure 4, one can detect only a few patterns that are present in every case, *i.e.*, C-C-A at the receptor end and A-G-C-Ψ-T-G in the left hand loop. In all other areas, there are a sufficient number of differences to permit the possibility of specific enzyme binding. If the clover leaf structures shown are correct, one would expect that in the paired stretches, the bases would be turned inward and inaccessible to enzyme recognition leaving the non-pairing stretches accessible. There are abundant differences even there. To date no sequences have been established for non-yeast tRNA's, but it is obvious that some differences must exist, for *E. coli* tRNA contains substantial amounts of thiouridine which is not found in yeast tRNA.

One must conclude that all tRNA's specific for a particular amino acid contain one or more features in common and that variations in the ability to be aminoacylated with heterologous enzymes reflect either differences in the binding of tRNA to enzyme or binding in a non-productive mode. In either case, these variations must be due to structural variations outside the enzyme recognition area of the tRNA and to differences in the corresponding parts of the enzymes.

Surprisingly, the cross reactions are not at all reciprocal. Thus, starfish leucine enzyme esterifies *E. coli* tRNA excellently while *E. coli* leucine enzyme appears inert towards starfish tRNA. Both yeast enzymes are active towards *E. coli* tRNA while the reverse pair do not react well. Toadfish tRNA is very active with yeast leucine enzyme while the reverse pair is sluggish.

Some generalization may be valid. Both of the *E. coli* ligases discriminate strongly in favor of homologous (*E. coli*) tRNA while *E. coli* tRNA frequently reacts well with ligases from other sources. Species specifically in these reactions appears to reside more in the enzymes than in the tRNA's, perhaps a consequence of great inflexibility of some of the enzymes.

Summary

1. Amino acid activating enzymes (aminoacyl tRNA ligases) specific for valine and leucine tRNA have been partially purified from four widely differing phyla, namely yeast, *E. coli,* starfish and toadfish.

2. The rates of aminoacylation of the four tRNA's have been determined using both the homologous enzyme and the three heterologous enzymes. In most cases there was appreciable cross-reaction, the *E. coli* enzyme and tRNA being in general least disposed to cross-react with the others.

3. In some cases the heterologous pair react more rapidly than the homologous. In one case this has been established as being a consequence of a much higher V_{max} that overcomes a poorer enzyme tRNA association.

4. Both homologously and heterologously esterified tRNA's appear to be homogeneous as measured by the kinetics of hydrolysis.

5. We interpret these observations as indicating that all tRNA's specific for a particular amino acid have at least one common polynucleotide sequence, probably in an area of the chain which is not base-paired. The marked differences in the rates of esterification and in the rates of hydrolysis of the aminoacyl tRNA's reflect other structural variations in tRNA's that do not include the enzyme recognition site. In particular *E. coli* enzymes are least able to adapt themselves to heterologous tRNA's.

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